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<b>13. ABSTRACT (Maximum 200 Words)</b> Estrogen can be metabolized to hydroxylated catechol estrogen, a genotoxic metabolite of estrogen, which causes DNA damage and tumors in animal models. In situ synthesis of estrogen in the breast through aromatase results in high tissue estrogen concentrations. We hypothesized that overexpression of aromatase in breast tissue increases tissue estradiol concentrations and consequent genotoxic metabolites, and eventually causes breast cancer. To test our hypothesis, we stably expressed aromatase cDNA in MCF-10A cells, a benign breast epithelial cell line. We have demonstrated that MCF-10A <sup>arom</sup> cells expressed functional aromatase. We demonstrated that MCF-10A <sup>arom</sup> cells expressed functional aromatase using tritiated water release assay and products isolation by thin layer chromatography. MCF-10A <sup>arom</sup> cells, incubated for 3 months with aromatase substrate, androstenedione, formed colonies in soft agar indicating the overexpression of aromatase induces cellular transformation. MCF-10A <sup>arom</sup> cells have all enzymes required to convert estrogen to catechoestrogens and quinine. Overexpression of aromatase enhanced production of genotoxic metabolites, which could be blocked by aromatase inhibitor, letrozole. We finished the long-term in vivo study. MCF-10A <sup>arom</sup> cells did not form tumors in nude mice.				
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## Introduction

Clinical data and results from animal studies indicate that estrogen not only promotes breast cancer growth but also plays a role in breast cancer initiation. The carcinogenic effect of estrogen is mediated by hydroxylated catechol estrogen, a genotoxic metabolite of estrogen, which causes DNA damage (1-4). In situ synthesis of estrogen in the breast through aromatase makes the major contribution to the high tissue estrogen concentrations (5). We hypothesized that overexpression of aromatase in breast tissue increases tissue estradiol concentrations and consequent genotoxic metabolites, and eventually causes breast cancer. To test our hypothesis, we have established a stable cell line of benign breast epithelial cell by expressing aromatase gene in MCF-10A cells. During this annual report period, we measured estrogen metabolites and depurinating DNA adduct in aromatase expressing MCF-10A cells (Specific Aim 2); fulfilled the in vivo tumorigenesis studies (Specific Aim 3) and carried out an assay for loss of heterozygosity (Specific Aim 3).

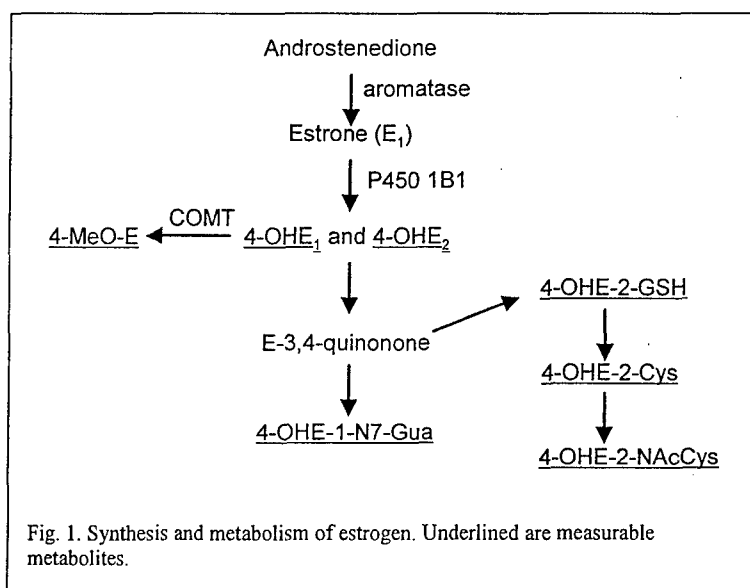
## Body

### I. In vitro study

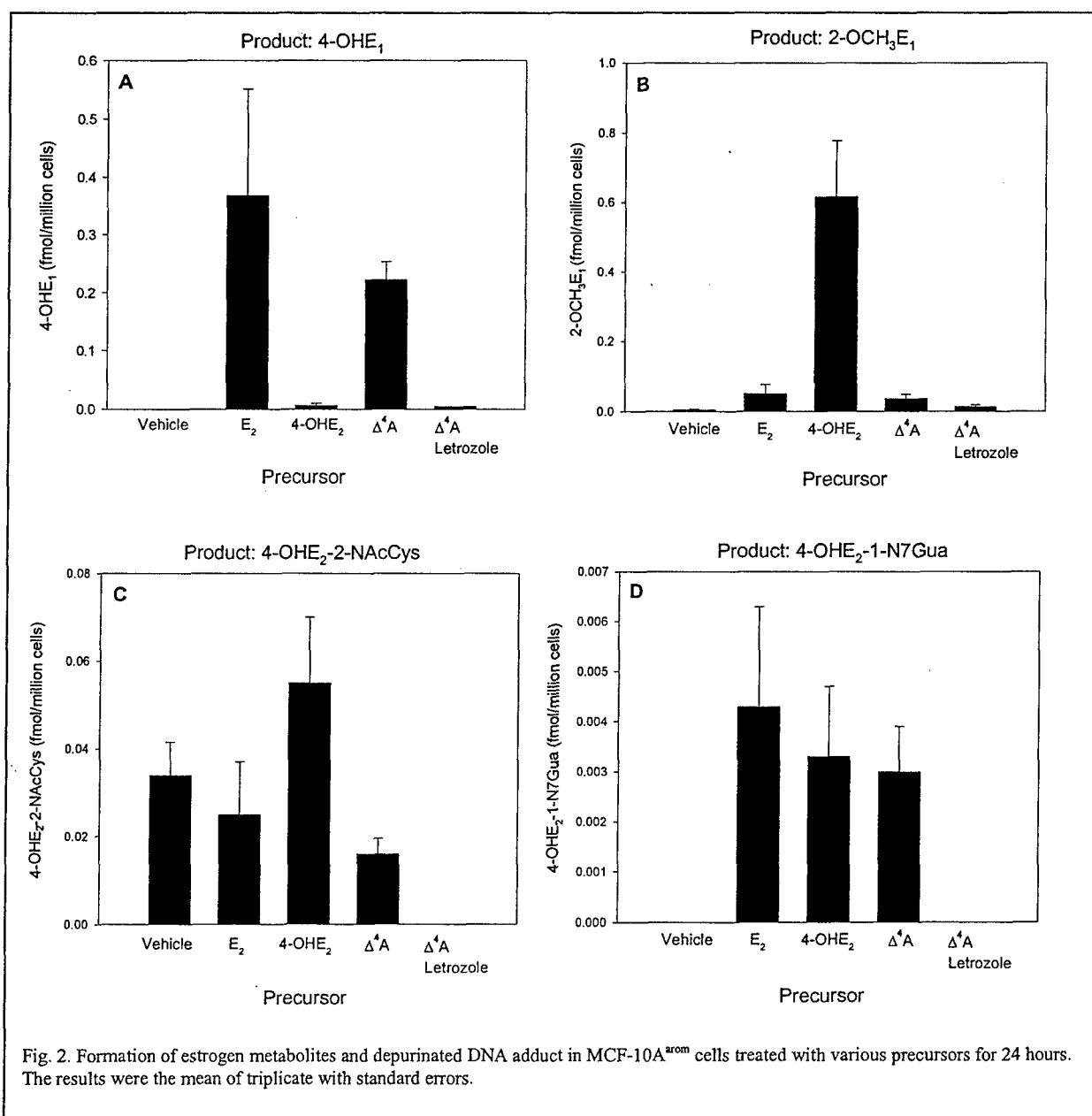
#### 1. Metabolite measurement

MCF-10A<sup>arom</sup> and MCF-10A<sup>vect</sup> cells were cultured in 150 mm dishes until confluence. The cells were treated in serum free medium with vehicle, estradiol, 4-OHE<sub>2</sub>, androstenedione plus or minus letrozole for 24 h. All compounds were at the concentration of 10  $\mu$ M. Cells and media were collected and kept at -80C until analysis. Estrogen metabolites, conjugates, and depurinated DNA adducts were measured by HPLC with a 12-channel electrochemical detector and confirmed by mass spectrometry (6-8). Figure 1 illustrates the metabolic pathways of estrogen and measurable metabolites/DNA adducts.

MCF-10A cells have all enzymes that catalyze conversion of estrogen to catechoestrogens and their methylation (Fig. 2). Incubation of MCF-10A<sup>arom</sup> cells with estradiol (10  $\mu$ M) for 24 hours dramatically increased production of 4-hydroxyestrone (Fig. 2A). Incubation with aromatase substrate, androstenedione ( $\Delta^4$ A), also increased the level of 4-hydroxyestrone (Fig. 2A) indicating that  $\Delta^4$ A was aromatized to estrogen which was metabolized to 4-hydroxyestrogen. A large portion of catechoestrogens were converted to methoxyestrogens,



detoxification metabolites, by catecho-*o*-methyl transferase (COMT). This is more significant when 4-OHE<sub>2</sub> was used as a precursor (Fig. 2B). Estrogen-3,4-quinones (E-3,4-Q) are the core genotoxic metabolites of estrogens. Estrogen-3,4-quinones are highly reactive and form conjugates with glutathione or bind to purine motifs on DNA as soon as they form (Fig. 1). Formation of glutathione conjugates and their metabolites reflects production of E-3,4-Q. As shown in Fig. 2C, 4-OHE<sub>2</sub>-2-NACys, a conjugate of E-3,4-quinone, was detected in MCF-10A<sup>arom</sup> cells pretreated with vehicle, E<sub>2</sub>, 4-OHE<sub>2</sub>, and Δ<sup>4</sup>A. Depurinated DNA adduct, 4-OHE<sub>2</sub>-1-N7Gua, was only detected in the cells treated with E<sub>2</sub>, 4-OHE<sub>2</sub>, and Δ<sup>4</sup>A. The level of 4-OHE<sub>2</sub>-1-N7Gua was undetectable in the cells incubated with Δ<sup>4</sup>A plus the specific aromatase inhibitor, letrozole (Fig. 2D).



## 2. Loss of heterozygosity (LOH)

Loss of heterozygosity may contribute to the initiation and progression of breast cancer. LOH has been observed in both in situ and invasive breast carcinomas on multiple chromosomal arms (9, 10) as well as in normal tissue adjacent to breast carcinomas (11). Human breast epithelial cells transformed with estradiol also showed LOH in chromosome 11 (12). To determine whether aromatase overexpression causes gene instability, LOH was assessed in MCF-10A<sup>arom</sup> cells in collaboration with Dr. Russo at Fox Chase Cancer Center, Philadelphia.

Cell lines: MCF-10A (regular MCF-10A), MCF-10A<sup>vect</sup> (transfected with empty pH $\beta$  vector) and MCF-10A<sup>arom</sup> cells.

Treatment: MCF-10A<sup>arom</sup> cells were treated for 10 months with androstenedione ( $\Delta^4$ A,  $10^{-6}$  M),  $\Delta^4$ A + letrozole ( $10^{-7}$  M), letrozole alone and estradiol ( $10^{-6}$  M). Regular MCF-10A and MCF-10A<sup>vect</sup> cells were without treatment.

Genomic DNA was extracted LOH was assayed using microsatellite polymorphism makers TP53dint and D13S893 that flank *p53* (chromosome 17) and *WT2* (chromosome 11p15.5) respectively. Both genes are tumor suppress genes.

All samples display one allele of 185 bp for the marker D13S893 and one allele of 295 bp for the marker TP53dint. It is necessary to examine more loci.

## II. In vivo study

To determine whether aromatase overexpression causes genotoxic damage and consequently mammary tumor, tumorigenesis of aromatase expressing MCF-10 cells were assessed in nude mice. Long term experiment for tumor formation and prevention was carried out during this report period.

Ovariectomized nude mice were inoculated subcutaneously with 5 million MCF-10A<sup>arom</sup> on left flank and regular MCF-10A cells on right flank. The animals were then divided into three groups, 20 mice each. One group of animals received androstenedione silastic capsule to provide aromatase precursor. One group of animals received androstenedione plus letrozole (5  $\mu$ g/day, s.c.). The third group served as a control. The mice were treated for one year. There was no palpable tumor developed from either regular MCF-10A or from MCF-10A<sup>arom</sup> cells.

## Key Research Accomplishments

- Determination of estrogen metabolites and depurinating DNA adduct in MCF-10A<sup>arom</sup> cells
- Loss of heterozygosity assay
- in vivo tumorigenesis

## Reportable Outcomes

An abstract entitled "Investigation of the carcinogenic effect of estradiol in the in vitro and in vivo models without estrogen receptors" was submitted to the International Conference of Aromatase 2004 for poster presentation in September 2004, Edinburgh, United Kingdom.

## Conclusions

MCF-10A<sup>arom</sup> cells have all enzymes that catalyze formation of catechoestrogen and methoxyestrogen. Expression of aromatase increases in situ estrogen synthesis and its genotoxic products, which can be blocked by aromatase inhibitor, letrozole. There is no LOH detected in the loci using TP53dint and D13S893 as markers. No tumor formed from MCF-10A<sup>arom</sup> cells in nude mice.

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## **Appendices**

Abstract submitted to the International Conference of Aromatase 2004.



Investigation of the carcinogenic effect of estradiol in the in vitro and in vivo models without estrogen receptors

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Estrogen causes cancers in animals. Substantial correlative data suggest that estrogens cause breast cancer in women. A body of emerging data suggests that estrogen is metabolized to genotoxic products that damage DNA and initiate cancer. This mechanism together with receptor-mediated stimulation of cell proliferation may act in concert to induce breast cancer. To test the genotoxic metabolite hypothesis, two models were employed: MCF-10A benign breast epithelial cells expressing aromatase were used to determine production of estrogen metabolites and their blockade by the aromatase inhibitor, letrozole. ER $\alpha$  knockout mice expressing the Wnt-1 oncogene (ERKO/Wnt-1) were chosen as an in vivo model to determine tumorigenesis in mammary glands where there is no functional ER $\alpha$  expressed. Incubation of MCF-10A<sup>arom</sup> cells with androstenedione (10  $\mu$ M) for 24 h resulted in an increase in the levels of 4-hydroxyestrone as well as its detoxicated product, 4-OHE-NAcCys, which was blocked by letrozole. These results indicate that breast epithelial cells have the enzymes that are required to convert estrogen to the genotoxic products. Overexpression of aromatase enhances in situ production of estrogen and its genotoxic metabolites. The ERKO/Wnt-1 mice were ovariectomized at 15 days. E<sub>2</sub> was administrated through s.c. silastic capsules. Predicted serum concentrations of E<sub>2</sub> delivered by silastic implants were 80 and 240 pg/ml. By 11 months, tumor incidences were 50% in animals receiving high dose E<sub>2</sub> and 25% with lower dose. In the control group only 16% of animals developed tumors (p<0.04). By 18 months, 66% of animals receiving high dose E<sub>2</sub> developed mammary tumors. Our data provide the first direct evidence that estrogen may cause breast cancer through a non estrogen receptor mediated mechanism.

Submitted to the International Conference of Aromatase 2004, Edinburgh, UK